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Studies on Formation of Liquid and Gaseous Formaldehyde-Induced DNA-Protein Crosslinks in Rat Marrow Cells

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Abstract: Formaldehyde (HCHO) is a genotoxicant which is suspected to have the ability to induce leukemia in epidemiology field. However, little is known about the possible mechanism how this agent induces leukemia. In this study, rat marrow cells are chosen to be the exposure targets to both liquid and gaseous formaldehyde. DNA-protein crosslinks are evaluated *via* KCl-SDS assay while single cell gel electrophoresis technique (comet assay) is used to test the DNA strand breaks of bone marrow cells. Our results show that low concentrations of formaldehyde (liquid: 5μmol·L^{-1} and 25μmol·L^{-1}, gaseous: 0.5mg·m^{-3} and 1.0mg·m^{-3}) can cause significant DNA breakage ($p<0.01$); the higher concentrations of liquid formaldehyde (liquid: 125μmol·L^{-1} and 625μmol·L^{-1}, gaseous: 3.0mg·m^{-3}) can cause significant DPC ($p<0.01$; $p<0.05$) compared with the control. The results suggest that formaldehyde can induce DNA damage in rat marrow cells which imply formaldehyde is highly possible to induce leukemia.

Keywords: formaldehyde; leukemia; genotoxicity; marrow cells; DNA-protein crosslinks; single cell gel electrophoresis; KCl-SDS assay

1 Introduction

Formaldehyde (HCHO) is a popular indoor-air which is proved to have genotoxic to human health. The wide exposure of formaldehyde, especially diffusing from certain new furniture, let this agent become more harmful to people. Researches show that occupational exposure to formaldehyde has been shown to induce nasopharyngeal cancer (IARC, 1995). Formaldehyde is also found to be mutagenic in human lymphoblasts at the thymidine kinas locus and HPRT loci (Crosby *et al.*, 1988). World Health Organization (WHO) has confirmed that formaldehyde is a factor which may cause cancer in respiration system and is suspected as a risk factor for children leukemia (IARC, 2004). Epidemiological studies have associated formaldehyde exposure with elevated risks for lymphohematopoietic system cancers at various sites (Hauptmann *et al.*, 2003; Pinkerton *et al.*, 2004). Soffritti *et al.* (1989) assumed that high doses of formaldehyde in the drinking water did induce leukemia in Sprague-Dawley rats. The International Agency for Research on Cancer (IARC, 2004) recently reevaluated formaldehyde and concluded that two recent studies provided "strong but not sufficient evidence for a causal association between leukemia and occupational exposure to formaldehyde."

The research of epidemiology showed that formaldehyde could cause leukemia. However, the possible mechanism how formaldehyde induces leukemia is uncertain. Lots of experiments suggested that exposure to formaldehyde induced several types of damage to DNA in cells, including double-and single-strand breaks (DSSB), DNA-DNA crosslinks (DDCs) and DNA-protein crosslinks (DPCs) (Orsière *et al.*, 2006). DPCs represent a significant form of DNA damage in formaldehyde-exposed cells (Orsière *et al.*, 2006). Increased number of DPCs has been found in the upper respiratory tract of monkeys and in the rat nasal mucosa (IARC, 1995; Feron *et al.*, 1991). Although DPCs can be removed, and the DNA can be restored during the proteolytic or the

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excision step of the repair process, failure to remove these lesions prior to cell division could cause cell death or mutation (IARC, 2004). The role of DPCs playing in initiating genotoxic in cells implies that the formation of formaldehyde-induced DPCs may be the possible molecular mechanism of how formaldehyde induces leukemia.

Blood stem cells, existing in marrow cells which can produce various blood cells, begin to produce blood cells at the initial embryonic stage, then proliferate and differentiate to produce various blood cells. Thereby marrow cells become the main blood-producing organs following the embryonic stage; meanwhile, blood stem cells possess the ability to make genetic stencils of themselves (Yang, 1997). Leukemia is a kind of malignant disease in blood systemic. Some researches showed that preleukemia is caused by anaplasia of blood stem cells, and that cause canceration which induces the unconventionality of blood-producing function in marrow cells, finally develop into leukaemia. In this study, we chose marrow cells to discuss the possible molecular mechanism of leukemia caused by formaldehyde based on this important relationship between marrow cells and leukemia (Yang, 1997).

2 Materials and methods

2.1 Reagents and apparatus

Reagents: 10% formalin was purchased from Sigma. Proteinase K was purchased from Merk. All other reagents were of the highest grade commercially available.

Apparatus: A 4160 type digital electrochemical analyzer (Interscan Inc., USA) was used to measure the concentrations of gaseous formaldehyde. WH-2 environmental chamber (Yuxin Technique Developing Inc., Wuhan) was used to generate gaseous formaldehyde. The chamber temperature ranged from 26℃ to 30℃, and the humidity was (45±5)%, and the gas flux of it was at (1±0.03)L·min^{-1}. Low temperature centrifuge (Eppendorf-5415R), Nikon fluorescence microscope (E600), Enzyme linked immunoassay detector (DG5031, Hua Dong Vacuum Tube Factory, China), and Fluorescence spectrophotometer (RF-5301PC, Shimadzu) were used in the experiments.

2.2 Methods

2.2.1 Liquid formaldehyde exposure method

Bone marrow cells were separated from Wistar male rats which were supplied by the Experimental Animal Center of Hubei. The rats were killed by cervical dislocation, and the tibiae were removed, freed of soft tissue. The distal end of the bone was removed, a hole was made in the proximal end, and the cells were flushed out with 37℃ PBS buffer. The cells were dispersed by repeated pipetting to give a concentration as about 1×10^6 cells·mL^{-1}. Then the suspended cells were distributed to Eppendorf tubes with sigh according to 0.5mL per tube. After that, the bone marrow cells were incubated for 1h in PBS medium at 37℃ with increasing concentrations of 0, 5, 25, 125, 625μmol·L^{-1} formaldehyde.

2.2.2 Gaseous formaldehyde exposure method

Totally 24 male Wistar rats were divided into 4 testing groups (n=6 each), and exposed to different concentrations of formaldehyde—0mg·m^{-3}, 0.5mg·m^{-3}, 1.0mg·m^{-3} and 3.0mg·m^{-3}. The inhaled groups were exposed to different concentrations of gaseous formaldehyde continuously for 72h, and were supplied with forage and water twice a day. After exposure, rats were sacrificed immediately.

2.2.3 KCl-SDS assay

The KCl-SDS assay was initially developed by Liu *et al* and modified by Zhitkovich and Costa in 1992 for detecting DPC in whole cells. In this study the KCl-SDS assay was based on Zhitkovich and Chakrabarti methods (Zhitkovich *et al.*, 1992; Chakrabarti *et al.*, 1999) with some modification to detect formaldehyde-induced DPC. In our experiments, the isolated cells were harvested by centrifugation at 6000rpm for 3min. The cells were resuspended in a 0.5mL of PBS, pH 7.5, followed by lysis with a

0.5mL of 2% SDS solution with gentle vortexing. The lysate solution was heated at 65℃ for 10min and then 0.1mL of pH 7.4 10mmol·L^{-1} Tris-HCl containing 2.5mol·L^{-1} KCl was added, followed by passing the resultant mixture six times through a 1-mL polypropylene pipette tip to favor shearing of DNA for a uniform length. The samples were placed on ice for 5min and were then collected by centrifugation at 10,000rpm for 5min. The supernatants were collected in different labeled tubes. The pellets (containing DPC)were washed three times by resuspending in 1mL washing buffer (0.1mol·L^{-1} KCl, 0.1mmol·L^{-1} EDTA, and 20mmol·L^{-1} Tris-HCl, pH 7.4)followed by heating at 65℃ for 10min, chilling in ice for 5min. The latter supernatants from each wash were added into the previous one with unbound fractions of DNA. The final pellet was resuspended in 1mL proteinase K solution (0.2mg·mL^{-1} soluble in a wash buffer)and digested for 3h at 50℃. The resultant mixture was centrifuged at 12,000rpm for 10min and the supernatant was collected (the supernatant contained the DNA previously involved in DNA-protein crosslinks). 1mL of either the supernatant containing the unbound fraction of DNA or the supernatant containing the DNA previously involved in DNA-protein crosslinks was then mixed with 1mL freshly prepared fluorescent dye Hoechst 33258(400ng·mL^{-1} soluble in a 20mmol·L^{-1} Tris-HCl), and then the tubes were allowed to stand for 30min in the dark. The sample fluorescence was measured using a RF-5301PC fluorescence spectrofluorimeter with excitation wavelength at 350nm and emission wavelength at 450nm. The DNA contents of the samples were determined quantitatively through a corresponding DNA standard curve generated from a set of calf thymus DNA. The DPC coefficient was measured as a ratio of the percentage of the DNA involved in DPC over the percentage of the DNA involved in DPC plus unbound fraction of DNA.

2.2.4 Comet assay(SCGE)

The protocol was performed according to Tice *et al*.(2000), with some modifications. Briefly, bone marrow cells were cast into miniature agarose gels on microscope slides and lysed in situ to remove DNA associated proteins and allow the compacted DNA to relax in Lysis buffer (2.5mmol·L^{-1} NaCl, 100mmol·L^{-1} EDTA, 10mmol·L^{-1} Tris-HCl (pH=10), 1% Triton X-100, and 10% DMSO). After lysis at 4℃ for 2h, proteinase K was added to the lysis solution (final concentration 10mg·mL^{-1})and additional lysis was performed at 37℃ for 2h. Following cell lysis, all slides were washed through three changes of deionized water at 20-min intervals to remove salt and detergent from the microgels. Slides were placed in a horizontal electrophoresis unit and were allowed to equilibrate for 20min with TBE buffer (300mmol·L^{-1} NaOH, 1mmol·L^{-1} EDTA, pH=13)before electrophoresis(17V, 240mA) for 20min. When electrophoresis was complete the slides were rinsed with water, air-dried, and stored protected from light until analysis.

2.3 Statistical analysis

All of the data was analyzed by software Origin 5.0. Student's t-test was applied to evaluate the significance of the differences in the results between treated and control groups. A level of $p<0.05$ was considered to be statistically significant.

3 Results

3.1 Liquid formaldehyde-induced DPCs in marrow cells

Fig.1 shows the DPC coefficient of rat marrow cells induced by liquid formaldehyde in different concentrations. As can be seen, comparing with the control group, the lower concentrations (5μmol·L^{-1} and 25μmol·L^{-1})exposure to marrow cells has no significant effect on DPC coefficient. However, there are significant differences between the higher exposure of formaldehyde (125μmol·L^{-1} and 625μmol·L^{-1})and the control group.

3.2 Gaseous formaldehyde-induced DPCs in marrow cells

Fig.2 shows the effect of gaseous formaldehyde-

induced DPCs in marrow cells with different concentrations. As the same trend with Fig.2, DPC percentage significantly increased when exposure to 3mg·m^{-3} formaldehyde. Lower concentration of formaldehyde (0.5mg·m^{-3}, 1.0mg·m^{-3})has the decreasing trend of DPC percentage when compared with control group.

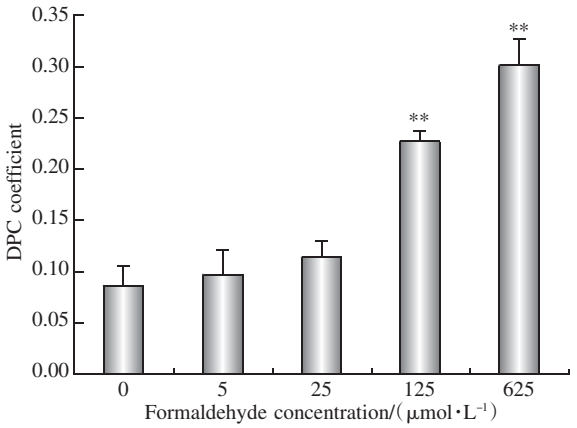

Fig.1　The effect of liquid formaldehyde-induced DPCs in marrow cells(**: p<0.01, formaldehyde treated groups compared with control group)

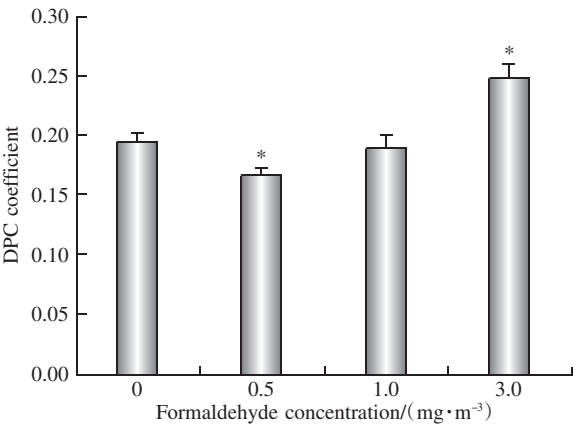

Fig.2　The effect of gaseous formaldehyde-induced DPCs in marrow cells(*: p<0.05, formaldehyde treated groups compared with control group)

3.3　Liquid formaldehyde induced DSSB

Fig.3 shows the tail DNA percentage and tail moment of rat marrow cells induced by liquid formaldehyde in different concentrations. As can be seen, comparing with the control group, the lower concentrations(5μmol·L^{-1}, 25μmol·L^{-1}, and 125μmol·L^{-1})exposure to marrow cells has significant effect on both tail DNA percentage and tail moment. However, higher concentration of formaldehyde (625μmol·L^{-1})has the decreasing trend of the tail DNA percentage and tail moment when compared with control group.

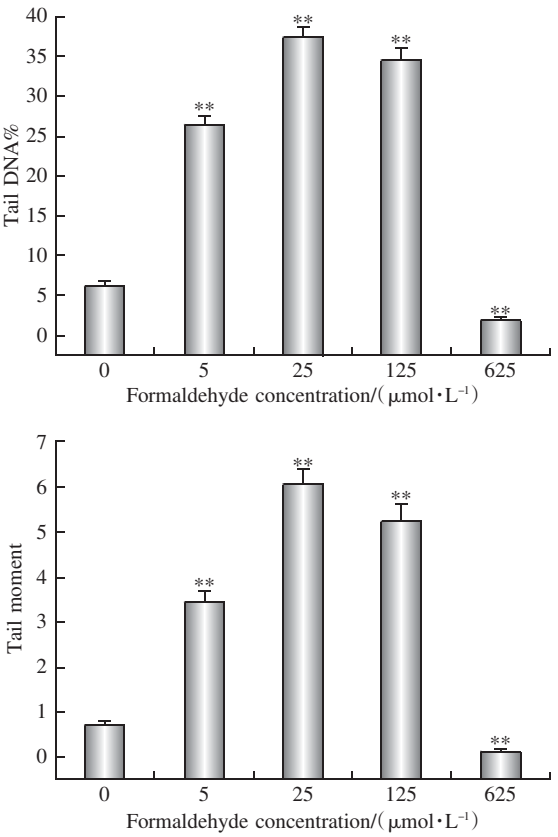

Fig.3　Tail DNA % and tail moment induced by liquid formaldehyde (**: p<0.01, formaldehyde treated groups compared with control group)

3.4　Gaseous formaldehyde induced DSSB

Fig.4 shows the effect of gaseous formaldehyde-induced tail DNA percentage and tail moment in marrow cells with different concentrations. As the same trend with Fig.4, both tail DNA percentage and tail moment significantly increased when exposure to 0.5mg·m^{-3}, 1.0mg·m^{-3}. Higher concentration of formaldehyde (3.0 mg·m^{-3})has the decreasing trend when compared with control group.

4　Discussion

In this study, the DPC coefficients and DSSB level were investigated in both liquid and gaseous formaldehyde exposure conditions. For DPC coefficients, Fig.1 and Fig.2 indicated clearly a dose-dependent relationship between the DPC coefficient and the concentration of formaldehyde. Both these two conditions got identical trend: there was no

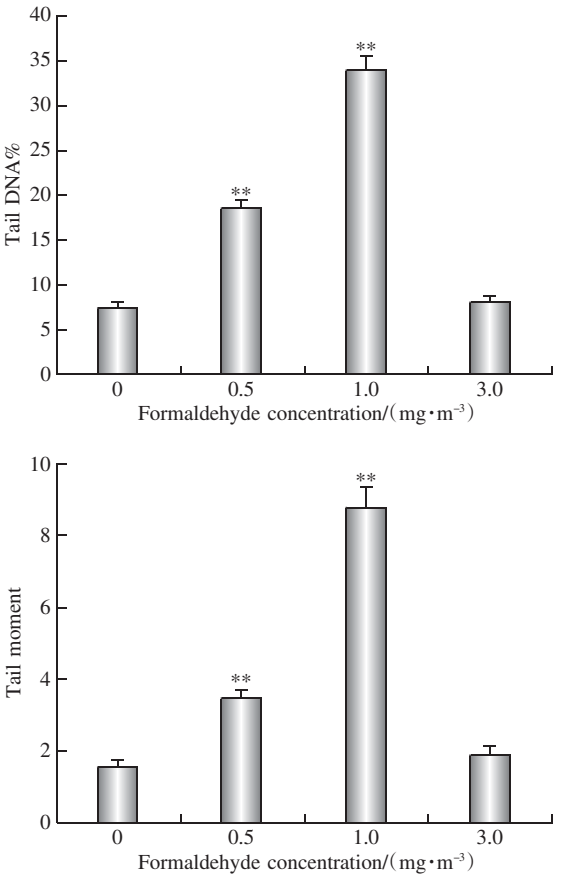

Fig.4 Tail DNA% and tail moment induced by gaseous formaldehyde (**: $p<0.01$, formaldehyde treated groups compared with control group)

significant difference in the DPC coefficient between the groups treated with relatively low concentrations (5, 25μmol·L^{-1} of liquid; 0.5, 1.0mg·m^{-3} of gaseous) formaldehyde and the control group. However, significant increase appeared between the groups treated with relatively high concentrations (125, 625μmol·L^{-1} of liquid; 3.0mg·m^{-3} of gaseous) formaldehyde and the control group ($p < 0.01$). The data described above suggested that relatively low concentration of formaldehyde could not induce DPCs in marrow cells while relatively high level formaldehyde could induce DPCs.

For DSSB level in bone marrow cells, low concentrations (5, 25μmol·L^{-1} of liquid; 0.5, 1.0mg·m^{-3} of gaseous) of formaldehyde could induce significant increases of DSSB comparing with the control group. Interesting, significant decreases or no significant difference were found between high concentrations (125, 625μmol·L^{-1} of liquid; 3.0mg·m^{-3} of gaseous) formaldehyde and the control group.

Combining all the data above, we suggest that a relatively low concentration range of formaldehyde can induce DSSB but can not induce the formation of DPC. By contract, DPCs can be induced under relatively high concentration while DSSB can not form in this concentration. Previous work suggested that at initial stage, blood stem cells are mutated gradually, further leading to the disfunction of marrow cells' ability to produce blood cells, and finally result in acute leukemia (AL) (Yang, 1997). If formaldehyde exposure in environment may induce DNA damage in marrow cells, it might result in the occurrence of leukemia. Our study showed that low concentrations of formaldehyde exposure could induce DNA breakage, and higher concentrations of formaldehyde exposure might lead to the formation of DNA crosslinks, which may have extensive genotoxicity on cells. Different concentrations of formaldehyde may induce different kinds and different extents of genotoxicity, and therefore might induce DNA damage and gene mutation in marrow cells, finally resulting in leukemia.

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液态或气态甲醛诱导大鼠骨髓细胞 DNA-蛋白质交联形成的研究

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摘要： 甲醛是一种遗传毒物，流行病学研究表明甲醛可能具有诱导白血病的作用，然而甲醛诱导白血病的机制目前还不清楚. 以不同浓度液态和气态甲醛对大鼠骨髓细胞进行染毒，采用KCl-SDS 法检测了骨髓细胞 DNA-蛋白质交联程度，并采用单细胞凝胶电泳技术(彗星实验)检测了骨髓细胞 DNA 链断裂程度. 研究结果表明：与对照组相比，低浓度甲醛(液态甲醛浓度为：5μmol·L^{-1} 和 25μmol·L^{-1}；气态甲醛浓度为：0.5mg·m^{-3} 和 1.0mg·m^{-3})可以引起 DNA 断裂水平显著增高 ($p<0.01$)；而高浓度甲醛（液态甲醛浓度为：125μmol·L^{-1} 和 625μmol·L^{-1}；气态甲醛浓度为：3.0mg·m^{-3})则可以引起 DNA-蛋白质交联水平显著增高($p<0.01$; $p<0.05$). 研究结果提示：甲醛染毒可以导致大鼠骨髓细胞 DNA 的损伤，暗示甲醛诱导白血病具有高度的可能性.

关键词： 甲醛；白血病；遗传毒性；骨髓细胞；DNA-蛋白质交联；单细胞凝胶电泳；KCl-SDS 测量法

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